

#39
Decl. w/attest
4/9/99
PATENT APPLICATION

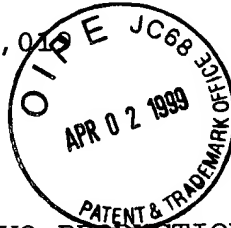
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Johnson et al.

Application No.: 08/458,019

Group Art Unit: 1651

Filed: 1 June 1995



Examiner: Lilling

For: PROCESSES FOR IN VIVO PRODUCTION OF
ASTAXANTHIN AND PHAFFIA RHODOZYMA
YEAST OF ENHANCED ASTAXANTHIN CONTENT

DECLARATION UNDER 37 CFR §1.131

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

We, the undersigned co-inventors, do hereby declare and state:

1. We are co-inventors of claims 25-34 currently pending in U.S. Ser. No. 08/458,109 and of the subject matter described therein.

2. Prior to 15 April 1988, the invention embodied in claims 25-34 of U.S. Ser. No. 08/458,019 was reduced to practice in the United States.

3. Prior to 15 April 1988, practicing the methods provided

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U.S. Appln. No. 08/458,019

in the above-captioned application, numerous strains of *Phaffia* which overproduced astaxanthin at levels above that found in wild type *Phaffia* were obtained, thereby attesting to the reproducibility of the methods and teachings described in the above-captioned application.

4. Thus, wild type or astaxanthin-overproducing strains of *Phaffia* were treated as taught in the above-captioned application to obtain strains producing higher and higher levels of astaxanthin.

5. The initial screenings of the strains often were visual because the feature of interest is a pigment which is manifest as culture color.

6. Prior to 15 April 1988, numerous strains were prepared, propagated as provided in the above-captioned application and then the amount of astaxanthin produced by each strain was determined as provided in the above-captioned application.

7. Attached hereto are copies of pages from laboratory notebooks depicting representative experiments demonstrating the reduction to practice of the invention as claimed.

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- a. Exhibit A is a copy of a page from a Kwok Ho laboratory notebook setting forth the results of astaxanthin content in Phaffia exposed to variety of treatments in shake flask cultures. The right-most column of figures --747, 865 and so on-- relate to astaxanthin content in micrograms per gram. All of the cultures contained astaxanthin at a concentration of more than 700 micrograms per gram.
- b. Exhibit B is a copy of a page from a laboratory notebook of Beril Geldiay-Tuncer setting forth the results of astaxanthin content in Phaffia astaxanthin mutants. Ant 1-4 is a deposited strain. As provided in the record, Ant 1-4 is identified as IGI887J2 in the application; K20 is identified as IGI887J1 in the application; and Ant 1-460 is identified as IGI2880B60 in the application. Numerous strains produced astaxanthin at a concentration of more than 700 micrograms per gram.
- c. Exhibit C is a copy of a page from a laboratory notebook of Beril Geldiay-Tuncer setting

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forth the results of astaxanthin content in Phaffia astaxanthin mutants. Ant 1-460 is identified as IGI2880B60 in the above-captioned application. Numerous strains produced astaxanthin at a concentration of more than 700 micrograms per gram.

d. Exhibit D is a copy of a page from a laboratory notebook of Beril Geldiay-Tuncer setting forth the results of astaxanthin content in Phaffia astaxanthin mutants. Ant 1-4 is a deposited strain. Ant 1-4 is identified as IGI887J2 in the application; K20 is identified as IGI887J1 in the application and Ant 1-460 is identified as IGI2880B60 in the application.

8. The dates redacted from the attached copies of laboratory notebook pages relating to testing various Phaffia strains for astaxanthin content are prior to 15 April 1988.

9. All of the activities described therein occurred in the United States.

10. We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information

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and belief are believed to be true; and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the instant application or any patent issuing therefrom.

Further Declarants sayeth not.

Date

Eric A. Johnson

Date

Huei-hsiung Yang

Date

Beril Geldiay-Tuncer

Date

William T. Hall

Date

David Schreiber

Date

Kwok Ho

APR-01-1999 03:29

P.06

PATENT APPLICATION

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Further Declarants sayeth not.

Date

Eric A. Johnson

Date

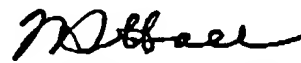
Huei-hsiung Yang

Date

Beril Geldiay-Tuncer

04.01.99

Date



William T. Hall

Date

David Schreiber

Date

Kwok Ho

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Further Declarants sayeth not.

Date

Eric A. Johnson

Date

Huei-hsiung Yang

Date

Beril Geldiay-Tuncer

Date

William T. Hall

Date

David Schreiber

4/1/99

Date

Kwok Ho

Kwok Ho

(H)

DECLARATION UNDER 37 CFR §1.131
 U.S. Appl. No. 08/458,019

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Further Declarants sayeth not.

 Date

April 1, 1999
 Date

 Date

 Date

 Date

 Date

 Eric A. Johnson

Huei-hsiung Yang
 Huei-hsiung Yang

 Beril Geldiay-Tuncer

 William T. Hall

 David Schreiber

 Kwok Ho

Phaffia Stock (5)

Block the

Purpose: Precursor & screen #2

Method: Cell both is from PHA125 (200L), age 49 hrs, 2.5mls were added to each flask,

Stock (2.5ml vol) (1.25ml added to each flask)

| | Dry wt. | T.I. (g/l) |
|--------------------------------|---------|------------|
| 1. soy oil 0.5ml | 39.3 | 747 |
| 2. soy oil 0.2ml | 32.7 | 863 |
| 3. DL methionine (1%) 0.05p | 26.7 | 1138 |
| 4. DL methionine (0.05%) 0.02p | 27.4 | 1113 |
| 5. Ac acetate (0.08%) 0.02p | 27.77 | 1201 |
| 6. Ac acetate (0.4%) 0.025p | 28.9 | 1052 |
| 7. Na propionate (0.05%) 0.02p | 24.9 | 1115 |
| 8. Na propionate (0.4%) 0.025p | 23.1 | 876 |
| 9. Safflower oil 0.3ml | 34.6 | 913 |
| 10. peanut oil 0.3ml | 33.3 | 846 |
| 11. leucine (0.2%) 0.05p | 27.4 | 1000 |
| 12. leucine (0.05%) 0.02p | 27.2 | 1143 |
| 13. Vitamin B12 0.5ml | 27.3 | 1154 |
| 14. control | 25.9 | 1158 |

Witness

Conducted by me,

Date

Reviewed by

Conducted by

Date

EXHIBIT A

E.

Book No.

29

Page 11.

Purpose: Comparison of astaxanthin titers within mutants K-20, and 1-4, BK2, ~~28~~ and Ant 1-428, and Ant 1-460 in YH broth.

| <u>Results:</u> | <u>I.D. Pan# Panug.</u> | <u>Pan+cells 0/5ml</u> | <u>A_{475nm}</u> | <u>pot vol (ml)</u> | <u>ASTAXANTHIN (mg/l)</u> |
|-----------------|-------------------------|------------------------|--------------------------|---------------------|---------------------------|
| 1- K-20 | | | 0.560 | 10 | 952 |
| 2- K-20 | | | 0.596 | 10 | 978 |
| 1- BK2 | | | 0.537 | 10 | 1081 |
| 2- BK2 | | | 0.573 | 10 | 1035 |
| 1- Ant 1-418 | | | 0.497 | 10 | 770 |
| 2- ant 1-428 | | | 0.441 | 10 | 870 |
| 1- ant 1-460 | | | 0.737 | 11 | 1608 |
| 2- ant 1-460 | | | 0.801 | 11 | 1678 |
| 1- ant 1-4 | | | 0.417 | 10 | 661 |
| 2- ant 1-4 | | | 0.425 | 10 | 670 |
| Ant 1-424 | | | 0.470 | 10 | 777 |
| ant 1-429 | | | 0.446 | 10 | 624 |
| ant 1-439 | | | 0.457 | 10 | 680 |
| ant 1-433 | | | 0.463 | 10 | 648 |

As seen above ant 1-460 is still pretty high. Astaxanthin culture of this brewer mutant will be prepared for the fermentation department

to Page 11a.

Witnessed Understood by me,

Date

Invented by

Benturina

Date

Recorded by

Benturina

EXHIBIT B

FLI

Screening

Book No.

31

Purpose: Screening of some of the selected mutant colonies of Ant 1-460 (NTG mutagenesis) in YM.

Incubation period 5 days.

Results: ml pet ether used for all the below was 20ml.

| <u>I.D.</u> | <u>Pan #</u> | <u>Pan (yp)</u> | <u>Pan + cell</u> | <u>glsalt brk</u> | <u>Amast (2)</u> | <u>Astma</u> |
|---------------------|--------------|-----------------|-------------------|-------------------|------------------|--------------|
| Ant 1-460 (variant) | 36 | 0.99609 | 1.02366 | .027 | 0.338 | 1192 |
| Ant 1-4601 | 30 | 0.99679 | 1.02822 | .031 | 0.357 | 1096 |
| Ant 1-4602 | 40 | 0.99640 | 1.02643 | .03 | 0.344 | 1092 |
| Ant 1-4604 | 33 | 0.99272 | 1.02488 | .032 | 0.402 | 1196 |
| Ant 1-4605 | 35 | 0.99151 | 1.02348 | .031 | 0.380 | 1167 |
| Ant 1-4606 | 38 | 1.00135 | 1.03178 | .031 | 0.359 | 1096 |
| Ant 1-4607 | 21 | 1.00558 | 1.03760 | .032 | 0.366 | 1077 |
| Ant 1-4608 | 37 | 1.79737 | 1.02785 | .030 | 0.421 | 1336 |
| Ant 1-4609 | 39 | 0.99705 | 1.02759 | .03 | 0.408 | 1295 |
| Ant 1-46000 | 31 | 1.00324 | 1.03239 | .034 | 0.353 | 974 |
| Ant 1-46011 | 34 | 0.99660 | 1.02753 | .03 | 0.345 | 1085 |

Ant 1-4608 and Ant 1-4609 looks promising
Check these back again.

Cleanliness of the strains here was tested
by plating some on to YM wright before extruding
of the 5 day old cultures.

to Page No.

| | | | | |
|---------|---------------------|------|-------------|------|
| Witness | I understand by me, | Date | Invented by | Date |
| | | | Recorded by | |
| | | | Bent Turner | |
| | | | Bent Turner | |

EXHIBIT C

Pigment formation w/ respect to time Book No.

32

Page No.

Purpose: To observe how and when the pigment astaxanthin is produced and does it decrease with time after a while. This has been a question because of variances we have been getting with 3 day old culture and 5 to 6 day old cultures, astaxanthin quantities.

Method: Inoculum: Frozen Aqueous Stock of BK2, Ant1-4, K-10, and 1-460

Medium YM in 500ml baffled flasks w/ 60ml volume.
10ml (5 for dry wt, 5 for astaxanthin)
Take sample on 1st, 5th and 7th day for quantification.

Results:

| TIME → I.D. ↓ | 48 hr. carement ↓ | 72 hr. centration ↓ | 144 hr. (ug/l) ↓ |
|---------------------|-------------------------|---------------------------|------------------------|
| BK2 | 623.91 | 850 | 1090 |
| K-20 | 635.80 | 914.9 | 1017 |
| Ant1-4 | 496.7 | 499 | 610 |
| Ant1-460 | 658.10 | 809.5 | 1509 |

Inoculum Understood by me.

Date

Inoculated by

Recorded by

Bert Kinner
Bert Kinner

Date

to Page No.

EXHIBIT D